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Bivalve mollusk circadian clock genes

can run at tidal frequency

Damien Tran^{1,2*}, Mickael Perrigault^{1,2}, Pierre Ciret^{1,2}, Laura Payton^{1,2}

¹University of Bordeaux, EPOC, UMR 5805, F-33120 Arcachon, France

²CNRS, EPOC, UMR 5805, F-33120 Arcachon, France

*Author for correspondence: Damien Tran

E-mail: damien.tran@u-bordeaux.fr

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SUMMARY

Marine coastal habitats are complex cyclic environments as a result of sun and moon interactions. In contrast to the well-known circadian orchestration of the terrestrial animal rhythmicity (~24h), the mechanism responsible for the circatidal rhythm (~12.4h) remains largely elusive in marine organisms. We revealed in subtidal field conditions that the oyster *Crassostrea gigas* exhibits tidal rhythmicity of circadian clock genes and clock-associated genes. Free-running experiment showed an endogenous circatidal rhythm. In parallel, we showed in the field that oysters' valve behavior exhibited a strong tidal rhythm combined with a daily rhythm. In the free-running experiment, all behavioral rhythms were circatidal, and half of them were also circadian. Our results fuel the debate on endogenous circatidal mechanisms. In contrast to the current hypothesis on the existence of an independent tidal clock, we suggest that a single "circadian/circatidal" clock in bivalves is sufficient to entrain behavioral patterns at tidal and daily frequencies.

INTRODUCTION

In a littoral zone, composed of intertidal and superior subtidal zones, multiple environmental cycles drive the behavior of the inhabiting species. In addition to the well-known circadian rhythm synchronized to the solar day at the origin of the light and temperature cycles, organisms have to deal and be in harmony with tidal cycles (12.4 h). Tidal cycles occurring once or twice per lunar day (24.8 h) are the result of the combination of the moon's orbit around the earth, for approximately 29.5 days (synodic lunar month cycle), and the 24 h earth rotation (solar day cycle). To anticipate and take advantage of environmental oscillations, marine organisms have developed endogenous mechanisms. Internal clock(s), nested in cells, enable organisms to account for time and are specifically synchronized by environmental cues associated with tides and solar day cycles [1–5]. Since the famous Cold Spring Harbor Symposium in 1960 [6], a debate persists on the existence and nature of one or several clocks to explain both circadian and circatidal rhythmicity [7]. Based on crustacean behavioral studies, three major hypotheses have been advanced (figure 1) to explain tidal rhythm. Naylor and colleagues [8] hypothesized the existence of two separate and unimodal circatidal and circadian clocks. A second hypothesis, by Palmer and colleagues [9,10], argued the existence of two unimodal circalunidian clocks (24.8 h lunar day cycle) coupled in antiphase. Finally, a third hypothesis advanced by Enright [11] suggested a single bimodal oscillator that governs both circadian and circatidal patterns. Unfortunately, most of these hypotheses are based on behavioral actograms of free-running (FR) experiments often characterized by high individual variability patterns as well as lability of rhythmicity, typical of the species living in the littoral zone [10]. Moreover, according to the time window and duration of the chronobiological analyses in FR experiments after entrained/field conditions, results can be differently interpreted to support the different hypotheses [4,7]. Since the 1980s for terrestrial organisms [12] and the 2000s for marine organisms [13], sequencing of clock genes has provided

remarkable progresses to understand circadian rhythms. Knockdown approaches to study circadian clock genes in the crustacean isopod *Eurydice pulchra* [14,15] and the mangrove cricket *Apteronomobius asahinai* [16,17] have provided strong results indicating that the circatidal rhythm is independent of the circadian rhythm. However, although the results of these studies are robust, these findings do not definitively rule out the possibility of another driving mechanism. Moreover, no specific circatidal clock gene has been cloned in any species to date.

Bivalve mollusks such as the oyster *C. gigas* occupy a key position in marine benthic food webs in terms of interactions with pelagic phytoplankton food resources [18]. *C. gigas* exhibits the largest geographic distribution among shellfishes likely related to the extensive exploitation of oysters for human consumption. Locally, *C. gigas* could be considered as an invasive species and represents a threat to ecosystems [19]. This mollusk inhabits intertidal and superior subtidal zones, making this species a key model to investigate mechanisms underlying the multiple rhythms of littoral organisms. Recently, the complete genome of this species was published [20]. Moreover, circadian clock genes and the cyclic transcriptome have been studied in *C. gigas* [21,22], making it an ideal candidate to investigate molecular clockwork and test hypotheses to explain circatidal rhythmicity.

MATERIALS AND METHODS

Experimental model and field details

All investigations were performed on Pacific oysters, *C. gigas*, of comparable age (approximately 1.5 years old, 65-75 mm shell length). All oysters were from natural recruitment in Arcachon Bay. Before each experiment, oysters were translocated and kept

either at the study site (Eyrac pier, Arcachon Bay, France, latitude 44.66°, longitude -1.16°) or at the Marine station of Arcachon (Arcachon Bay, France) with a continuous flow of natural sea water from the bay and a natural photoperiod. Detailed conditions are available in methods S1, table S1 and figure S1. All procedures using bivalves were approved and carried out in accordance with international ethical standards and French guidelines of the universities.

Valve activity behavior

For the valve activity behavior in the field experiment, 15 oysters were placed in a bag fixed on a permanently immersed oyster table on March 5, 2018. The valve activity behavior of these oysters was recorded for 18 days (March 5 to 23, 2018, 00 corresponds to 17 h, local time, UTC +1) using a high-frequency noninvasive (HFNI) valvometer field technology [23]. For the free-running (FR) behavior laboratory experiment, 14 oysters were pre-equipped with lightweight electrodes and placed in the study site (subtidal conditions, Eyrac pier, Arcachon Bay, France) for 3.5 weeks. Then, the oysters were placed in the laboratory under constant darkness (dark-dark, DD) and homogenized filtrated seawater, to record valve behavior with HFNI valvometer laboratory technology over 7 days. Detailed of HFNI valvometry technology and behavioral studies conditions are available in methods S1. Hourly individual VOA data from field and laboratory experiments are accessible in dataset S1.

Sampling for gene expression

For the gene-expression field experiment, 112 oysters were placed in the study site in 16 oysters bags. Gill tissues were sampled from 7 oysters every 3.1 h. The experimental sampling details are in table S1. The oysters were quickly dissected (2-3 min) in a shelter on

the Eyrac pier under dim red light during the night. Gill tissues were immediately and individually transferred into 700 μ L of RNA lysis buffer (Kit SV Total RNA Isolation System, Promega) with 0.5 g of ceramic beads and homogenized before storage at -80°C . The experimental protocols for the FR experiment (DD) and the experiment under LD entrainment are described in [21] and [22], respectively, from where raw data were extracted and used for the RAIN analysis. Details of sampling for gene expressions and experimental conditions are available in methods S1.

Gene expression

For the field study, total RNA from the gills was extracted from samples using SV Total RNA Isolation System kits (Promega). Total RNA quantity and quality were assessed by spectrophotometry and reverse transcribed using GoScriptTM Reverse Transcription System kits (Promega). Real-time qPCR reactions were performed on individual samples using the GoTaq^R qPCR Master Mix kit (Promega). For experiments in controlled environments (DD and LD), methods for total RNA extraction from the gills and gene expression quantification are explained in [21] and [22], respectively. Primer sets of circadian clock genes (*Cgclock*, *Cgbmall1*, *Cgper*, *Cgtim1*, *Cgcry2*, *Cgcry1*, *Cgrev-erb*, *Cgror*), clock-associated genes (*Cghiomt*, *CgRhodopsin 1, 2 and 3*) and housekeeping genes applied in this study are listed in table S3. The comparative Ct method $2^{-\Delta\Delta\text{Ct}}$ [24] was used to determine the relative transcript levels of candidate genes, where $\Delta\text{Ct} = \text{Ct}(\text{gene}) - \text{Ct}(\text{housekeeping gene})$. The mRNA sequences of the investigated genes can be found via the GenBank accession numbers given in table S3. Gene expression data from field studies, LD laboratory experiments and DD laboratory experiments are accessible in dataset S2. Detailed gene expressions procedure are available in methods S1.

Statistical and algorithmic analysis

Field and laboratory valve behavior data were analyzed with LabView 8.0 software (National Instruments). For valve behavior rhythmicity, chronobiological analyses were performed using TSA Serial Cosinor 6.3 software (Expert Soft Tech). Several steps were required to validate a significant rhythm [25,26]. Datasets of gene expressions were investigated for tidal and circadian periodicities in R [27] (32-bit, version 3.2.2) using the RAIN package [28]. The RAIN algorithm is a robust nonparametric method for the detection of rhythms of specified periods in biological data that can detect arbitrary wave forms. Circatidal/tidal periodicities were defined by a significant period range of 12.4 ± 3.1 h, and circadian/daily periodicities were defined by a significant period range of 24 ± 4 h.

To account for multiple testing of behavioral or genes expression, Benjamini-Hochberg adjusted p -values < 0.05 were considered significant [28]. Details of chronobiological analysis using Cosinor or RAIN are available in methods S1.

RESULTS

Bimodal valve activity behavior in the field

To determine oyster behavior in the natural environment, valve opening amplitude (VOA) was monitored by a high-frequency noninvasive (HFNI) valvometer biosensor at Eyrag, Arcachon Bay, France ($44^{\circ}66'N$, $1^{\circ}9'W$). This biosensor was deployed in March 2018 to record the behavior of 15 native oysters of Arcachon Bay that are exposed to tidal and daily cycles (table S1 and figure S1). In our subtidal conditions, the oysters were always submersed, avoiding drastically changing temperatures, oxygen levels and food supply at low

tides (~ 2 m under sea level depending on tide intensity). Valvometer data showed clear rhythmic VOA activities. Actograms (figure 2A) indicated a maximal VOA at high tide and a minimal VOA at low tide. A cosinor analysis of the mean VOA data (figure 2B) significantly validated a 12.4 h tidal rhythm correlated to the sea-level cycle, as well as a 6.2 h rhythm correlated to the maximum water current at each midebb and midflow of a tidal cycle. Moreover, oysters also expressed a significant but less pronounced 24.4 h daily rhythm. The individual VOA data (figure 2B, figure S2 and table S2) showed that 100 % of the oysters had a tidal rhythm of which 80 % were also bimodal (tidal and daily patterns). Mainly, the tidal component (12.4 and 6.2h cycles) expressed by the percent rhythm (PR) are more pronounced than the daily one (24h).

Bimodal and circatidal behavior in free-running conditions

To address the issue of the genetic origin of bimodal behavior, *C. gigas* individuals were collected at the same location and at the same time of year (March 2014). Oysters were placed immediately under constant darkness (DD conditions). The VOA behavior of 14 oysters was recorded during the 7 following days, and 85.7 % of the animals had significant rhythmic behavior (figure 2C, figure S3). At an individual level, 100 % exhibited a clear circatidal rhythm with a period of 13.31 ± 0.41 h (mean \pm SEM), whereas 50 % of the individuals also exhibited a circadian pattern with a period of 24.70 ± 1.32 h (mean \pm SEM).

Tidal expression of circadian clock genes in the field

To investigate the expression of the circadian clock genes in the field, oysters were collected at the same location as for the behavioral study (figure 3). Sampling was performed from March 14 to 16, 2018, close to the new moon to avoid any potential moonlight effect [29,30]. The experimental sampling details are in table S1. Transcriptional variations in the 8 circadian

core clock genes (figure 3A) as well as putative clock-associated genes (cags, figure 3B) were investigated (table S3) [21]. Among these candidates, *Cghiomt* is involved in the synthesis of melatonin [31], and three *rhodopsin-like genes* (*Cgrhodopsin-like 1, 2, 3*) are known to be associated with light perception [32]. The statistical analysis (figure 3C) using the RAIN algorithm [28], indicated a significant tidal rhythm for five circadian clock genes: *Cgclock*, *Cgbmall*, *Cgtim1*, *Cgror* and *Cgrev-erb*, which peaked during high tide slack. The cyclic expression of *Cgbmall* was bimodal, with a significant daily oscillation. Finally, *Cgcry1*, *Cgper* and *Cgcry2* exhibited only a significant daily rhythm. The peak expression for *Cgbmall* was nocturnal, whereas the peak expressions of *Cgper*, *Cgcry1* and *Cgcry2* were diurnal. In addition to the core circadian genes, all cags (*Cghiomt* and *Cgrhodopsin-like 1, 2, 3*) showed a significant tidal expression, with a peak of expression between high tides and ebb tides (figure 3B).

Circatidal endogenous oscillations and daily expression of circadian clock genes.

Raw data for clock gene expression from two previous experiments [21,22] were analyzed using the RAIN algorithm [28]. We first reanalyzed cyclic gene expression in FR conditions in DD from previous experiments [21] (figure 4A), and second, we reanalyzed clock gene expression in LD (9:15) entrainment, mimicking the photoperiod in winter, in laboratory experiment [22] (figure 4B). The statistical analysis (figure. 4C) using the RAIN algorithm showed that in DD, all core clock genes exhibited a significant circatidal profiles. However, three transcripts, *Cgclock*, *Cgbmall* and *Cgrev-erb*, showed bimodal expression with a significant circadian profile as well.

To verify that the circadian genes could oscillate at circadian frequency, their cyclic profiles were analyzed under LD conditions (figure 4C). We showed that all the clock genes

expression exhibited a strong daily pattern, except for *Cgball1*. No circatidal expression was significant for any transcripts.

DISCUSSION

In coastal and subtidal field conditions, the oyster *Crassostrea gigas* expresses bimodal valve behavior with a strong tidal pattern modulated by a daily rhythm. This bimodal phenotype has often been described in intertidal organisms, such as insects, crustaceans [4] and mollusks, such as the gastropod *Cellana rota* [33], but less for organisms settled in the subtidal zone of the littoral, excepted on the horseshoe crab *Limulus polyphemus* [34]. These results that oyster behavior exhibits both tidal and daily rhythms were validated by previous one-year studies at the same field location [25,35]. In contrast, in the absence of strong tidal forces in the Mediterranean Sea, the mussel *Pinna nobilis* expresses cyclic behavior related only to light entrainment [36]. Results from free-run experiments show that either circatidal or bimodal (circatidal and circadian) behavioral rhythms are observed in dark/dark (DD) conditions. This result for *C. gigas* is confirmed by previous results by Perrigault and Tran [21]. Bimodal swimming activity under DD conditions has also been reported in the isopod *E. pulchra* and is characterized by robust circatidal behavior, modulated by stronger activity during the subjective night [14]. The persistence of circatidal and circadian phenotypes in *C. gigas* valve activity behavior under constant conditions clearly shows an overt underlying clock mechanism(s) for both rhythms. However, these phenotypes do not predetermine the nature of the mechanism(s) that may involve one or two independent clockworks. Thus, we tested the hypothesis that a unique clockwork is able to generate both circatidal and circadian rhythms. To test this hypothesis in ecological field conditions [37,38], we studied the ability of circadian core clock genes to oscillate at tidal frequency. The expression of core circadian

clock genes and putative clock-associated genes (cags) was analyzed in the same field location as the behavioral study. It turns out that in natural conditions, five of the eight circadian clock genes express significant tidal rhythmicity, as well as the four analyzed putative cags. Interestingly, one of the clock genes (*Cgbmal1*) showed both tidal and daily expression patterns. Finally, the three other clock genes exhibited only daily oscillations. The proteins *CgCLOCK* and *CgBMAL1* form a heterodimer that acts as the principal transcriptional activator in the clock mechanism [39]. If *Cgclock* runs at tidal frequency and *Cgbmal1* is bimodal, then this scenario suggests that the protein complex *CgCLOCK-CgBMAL1* could have bimodal action on generated rhythms allowing either a circatidal or circadian rhythm or both for the gene expression under their control [40]. According to the model proposed for the oyster *C. gigas* clockwork [21], which is closely related to the monarch butterfly *Danaus plexippus* clockwork [41], the protein *CgCRY2*, associated with *CgPER* and *CgTIM1*, could act as a transcriptional repressor of clock functioning. *Cgcry2* and *Cgper* have daily expression, whereas *Cgtim1* is tidally expressed. We propose that the protein heterotrimer *CgCRY2-CgPER-CgTIM1* could also have a bimodal circadian/circatidal pattern to its transcriptional repressor action on *Cgclock* and *Cgbmal1*. The protein REV-ERB is known to repress the action of the positive transcriptional factor ROR on *bmal1* expression [42,43]. Our findings suggest that tidal oscillations in *Cgrev-erb* and *Cgror* could contribute to the tidal pattern of *Cgbmal1* expression. Finally, the weak diurnal *Cgcry1* oscillations is consistent with its putative involvement in light synchronization of the clock [44] and consequently with the weak daily rhythm of VOA showed figure 2. To validate the transcriptional regulatory actions of each clock gene, functional approaches are necessary. Unfortunately, the use of the *Drosophila* S2 cell transcriptional assay [14] is adapted for light entrainment experiments but not for investigations of the effects of tidal zeitgeber. The knockout gene technology, using CRISPR-Cas9 [45] gene-editing, is not currently operational

for bivalves. Finally, gene interference that allows the knockdown of clock gene expression [46] does not give a clear and definitive response, as gene transcripts that might be sufficient to maintain the transcriptional feedback loops of the clock machinery always remain.

Cghiomt is a homolog of the mammal *hiomt*, which is involved in the final step of melatonin synthesis [31]. Melatonin, the hormone of the day/night cycle, is under control of the circadian clock and plays a key role in the circadian rhythms of many physiological and hormonal processes [47]. Melatonin could also have a feedback effect on the clock as an inhibitor of the proteasome associated with the degradation of clock transcriptional factors [48]. Tidal expression of *Cghiomt* may shape the temporal action of melatonin in a tidal manner. Similarly, a tidal rhythmicity of the *Cgrhodopsin-likes* 1-3, that are homologs of the rhodopsin involved in light signal transduction [32], is observed. One can be argued that this tidal rhythmicity is explained by a tidal periodicity of light availability to the gills inside the palleal cavity, as a result of the valve opening tidal rhythm.

In the free-running experiment, we showed a clear endogenous circatidal rhythm for all clock genes studied, of which three genes also had circadian rhythms. Our results clearly indicate that in the absence of zeitgeber, clock genes could run at tidal frequency and bimodal patterns were exhibited for some of the genes. In comparison, only *tim1* (*EpTim*) in the head of the crustacean *E. pulchra* expressed a circadian rhythm under DD exposure, whereas other clock genes did not express cyclic patterns [14]. However, the profile of *EpBmall* strongly suggests a ~12 h circatidal expression that should be confirmed by statistical analysis. To control the ability of these circadian clock genes to run on a daily rhythm, we placed the oysters in light/dark (LD) entrainment conditions, which showed that *C. gigas* clock genes can express daily oscillations. These results suggest that oysters should have only a daily pattern in field conditions without tidal effects, such as in the Mediterranean Sea. Our data indicate that the

circadian clock can run at a tidal frequency as well as at a daily frequency according to the field locations and could be the origin of bimodal behavior in oysters.

Controversies and hypotheses still exist in terms of explaining the tidal rhythms of organisms living in the coastal environments of oceans [1–10]. Three major hypothesis are opposed based either on the existence of two unimodal clocks or either on a single bimodal clock (figure 1). In this study, we demonstrated that some clock genes are tidally expressed when oysters are subjected to both tidal and daily environmental cues occurring in the field. This finding suggests that a single clockwork could be entrained by two different zeitgeber, i.e. light/dark synchronization for the daily rhythm and mechanical vibration and/or water pressure for tidal rhythm [49].

Recent findings on crustaceans and insects [14,16,17] note the existence of two independent circadian and circatidal clockworks that could share some components, such as casein kinase ϵ . This assumption is based on the fact that interference of circadian clock gene expression (dsRNAi) lowers or removes the circadian rhythmicity but not circatidal rhythmicity in FR experiments. Despite their merits, the limits of these studies are that RNAi applications lead to a decreased-but-not-abolished expression of the targeted genes [14,16,17]. These knockdown studies do not preclude the possibility that interference in clock gene expression was not sufficient to affect tidal oscillation. Depending on the species and the ecological niches they occupy, circadian rhythm is likely more labile than circatidal rhythm. Thus, the partial knockdown of the clock gene would at least partially disrupt the circadian pattern but would have no effect or less of an effect on the tidal pattern, which would be more robust in the littoral species. Finally, although the strong arguments of this study in favor of a bimodal clock, the possibility to have a “master tidal clock” that drives a “slave circadian clock” to run at tidal frequency might be raised. To validate or refute the unimodal tidal clock hypothesis (dissociated from the circadian one), the use of a complete knockout technique by

mutagenesis, whose availability is still limited in marine organisms, might be a promising approach.

Conclusions

In natural conditions, we tested the hypothesis that the tidal rhythm of oysters might be driven by the circadian oscillator. Our results showed that the circadian clock genes could run at tidal frequency. This finding suggests that a single clock strategy could be selected to manage and interconnect the multi-temporal organization of biological processes in marine organisms. Integrating the tidal cues with the daily cues might occur in a single clock that would give bimodal or unimodal oscillation outputs according to the balance between tidal and daily cues in each specific location inhabited by oysters. However, no one can assume that all marine species share the same circatidal timing mechanism. Evolutionary selection across phyla could have resulted in the existence of separate clocks that share some components or single clocks that interact with different intermediate cogs and gears dedicated to the specific rhythms. Since the first ancestral clock emerged in aquatic environments where organisms first appeared [50], clockworks have faced both tidal and daily cues. In oysters, efficient evolutionary processes have been selected to keep the same components to deal with both rhythms.

Ethics

This paper meets the requirements set out in the Royal Society publishing ethics and policies criteria.

Data accessibility

The mRNA sequences of the investigated genes can be found via the GenBank accession numbers given in table S3. Hourly individual VOA data from field studies and laboratory experiments are accessible in Dataset 1. Gene expression data from field studies, LD laboratory experiments and DD laboratory experiments are accessible in Dataset 2.

Author contributions

DT was the principal investigator and performed the study design, fieldwork, laboratory experiments, behavioral analysis, rhythmic analysis, interpretation and manuscript preparation and review, funding. LP performed the study design, fieldwork, laboratory experiments, behavioral analysis, gene expression analysis, rhythmic analysis, interpretation and manuscript preparation and review. MP performed the study design, fieldwork, laboratory experiments, gene expression analysis, interpretation and manuscript review. PC performed the study design, fieldwork and manuscript review.

Competing interests

The authors declare no competing interests

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FIGURE LEGENDS

Figure 1. Three main molecular clock hypothesis proposed to explain tidal rhythm of organisms living in littoral area. In the yellow rectangle, the tested hypothesis using the mollusk bivalve, the oyster *Crassostrea gigas*.

Figure 2. Valve activity to determine tidal and daily influences on oyster behavior. (A1).

Colored panel shows a double-plotted actogram of the field mean valve opening amplitude (VOA) on 15 oysters at 4 m depths (\pm tides) over 18 days in Arcachon Bay, France. On the left, tide amplitudes are represented by sea level during this period. Black and half-black circles represent the new moon and the third quarter moon, respectively. White dashed lines on the actogram represent the high tides. The rectangle shows the period of gene expression sampling times. Experimental conditions, sunset, sunrise, moon illumination and position, sea level, light intensity and water temperature are detailed in table S1 and figure S1. (A2).

Chronobiological analysis of mean valve behavior during the field experiment. Time Series Analysis cosinor analysis was used to detect tidal periodicities (12.4 h and 6.2 h periods) and daily periodicity (24 h). Significant rhythms were determined by the periods found with Lomb and Scargle periodograms (above the dotted line, corresponding to p -value > 0.95 , peaks of significant periods T, the power corresponds to the intensity of the period) and by the adjusted p -value of the cosinor model. PR corresponds to the percent rhythm (%) of the model. The 2nd periodicities correspond to the second significant periodicities after residue injections. (B).

Field chronobiological analysis of individual oyster valve opening amplitudes. Right panel. Percentage of rhythmic oysters ($n = 15$) expressing solely a tidal pattern or expressing at time a tidal and a daily pattern. Left panel. Mean percent rhythm (i.e. the percentage of rhythmicity explained by the Cosinor model) according to the tidal periodicities (12.4 ± 0.5 and 6.2 ± 0.2 h) and the daily one (24.0 ± 1.0 h). Different letters represent significant differences at

adjusted p -value < 0.05 (One-Way ANOVA test and Bonferroni post-hoc test). Detailed analysis are presented in figure S2 and table S2. (C). Rhythmic analysis on individual VOA over 7 days under lab free-running condition (constant darkness, DD). Right panel. TSA cosinor analysis was used to detect a significant rhythm ($n = 14$ oysters, R: rhythmic individuals ($p < 0.05$), AR: arrhythmic individuals). Left panel. Percentage of rhythmic individuals with solely circatidal periodicity (~ 12.4 h) or expressing at time a circatidal and a circadian periodicity (~ 24 h). Individual profiles and periodograms are detailed in figure S3.

Figure 3. Cyclic expression patterns of *C. gigas* clock genes in the field. Temporal profiles of core clock genes (A) and clock-associated genes (B) mRNA levels were measured in *C. gigas* gills sampled in the field. Gray areas indicate night phases. Dark blue lines indicate sea level above oysters in meters (tides) on the right axis. Values are expressed in relative mRNA levels, means \pm SEM, $n = 5-7$ individual replicates per time point (16 sampling times every 3.1 h for 46.5 h). For clock genes full names, see table S3. Data were analyzed for rhythmic expression using the R package RAIN. The blue T indicates significant tidal rhythm (~ 12.4 h), and the red D indicates significant daily rhythm (~ 24 h). Stars indicate power of significance: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. (C). Inserted table of exact adjusted p -values using RAIN analysis. For details of the experimental sampling and environmental conditions, see table S1 and figure S1.

Figure 4. Cyclic expression of *C. gigas* core clock genes in controlled conditions.

Temporal profiles of clock gene mRNA levels were measured in *C. gigas* gills sampled in the laboratory (A) under constant darkness (DD) and (B) Light/Dark alternating regimen 9 h/15 h. Gray areas indicate darkness. Values are expressed as relative mRNA levels, means \pm SEM, n

= 9 oysters per time point in the DD and n = 5-6 oysters per time point in LD. For clock genes full names, see table S3. The blue CT indicates significant circatidal rhythm (~12.4 h), and the red CD/D indicates significant circadian/daily rhythm (~ 24 h). Stars indicate power of significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (C) Inserted table of exact adjusted p -values using RAIN analysis for DD and LD experimental data.

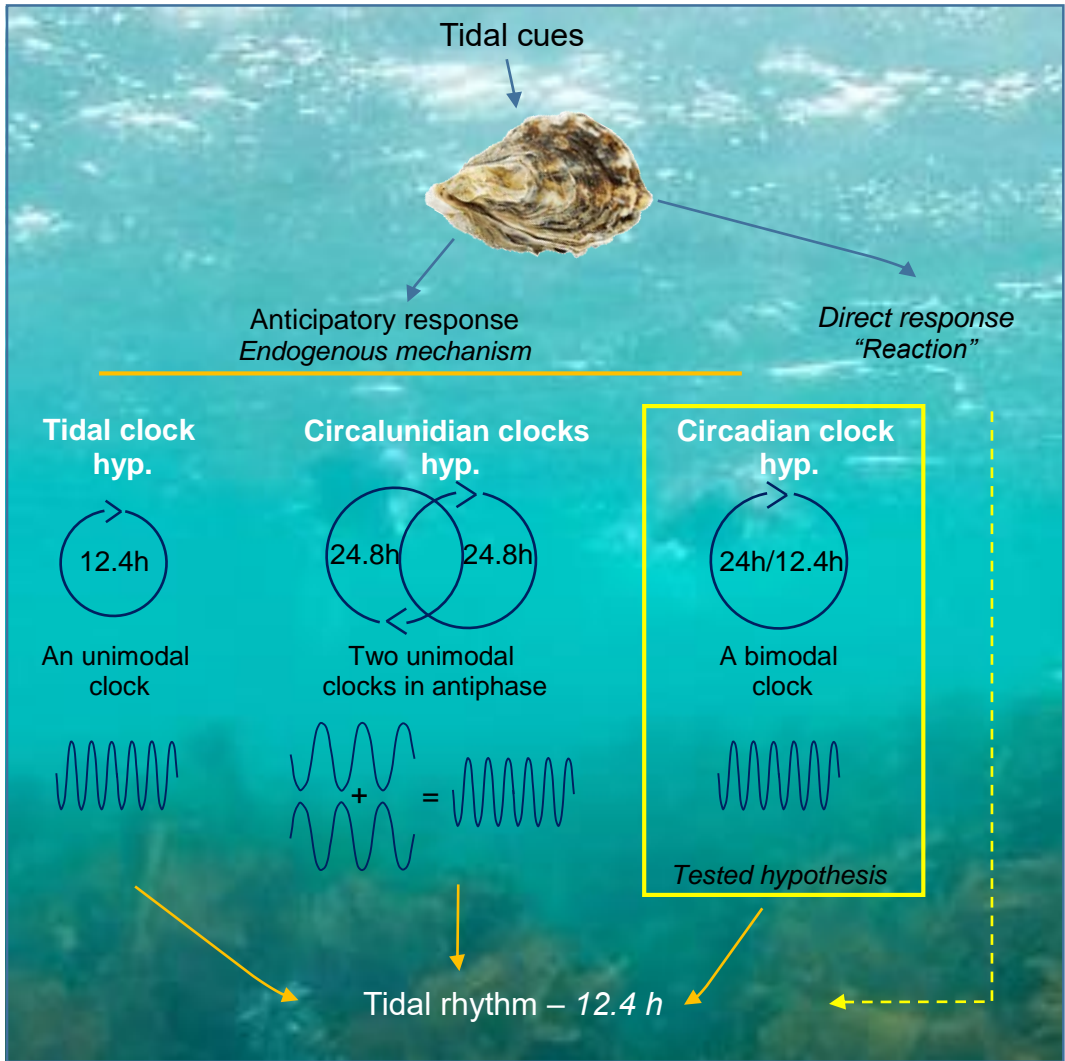
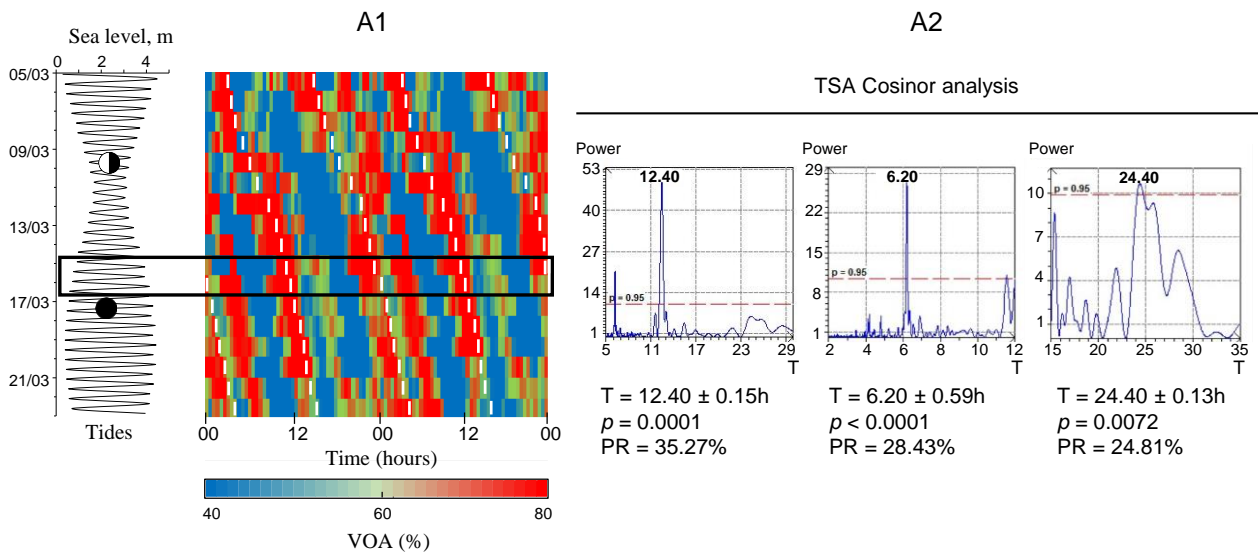
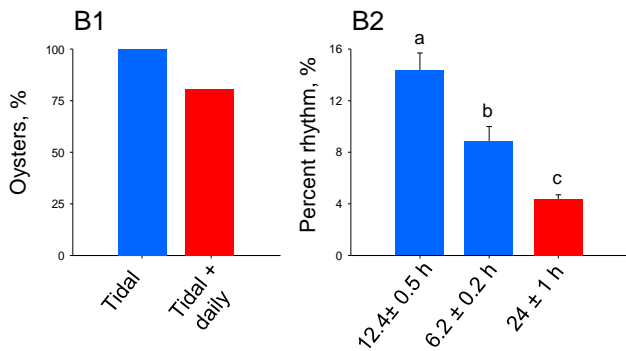


Figure 1.

A – Field rhythmic behavior – group level



B – Field rhythmic behavior – individual level



C – Free-running rhythmic behavior

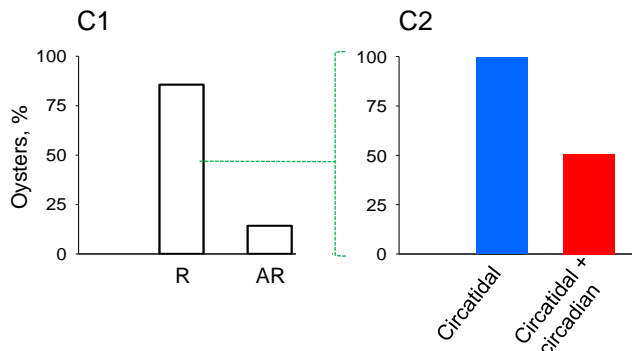


Figure 2.

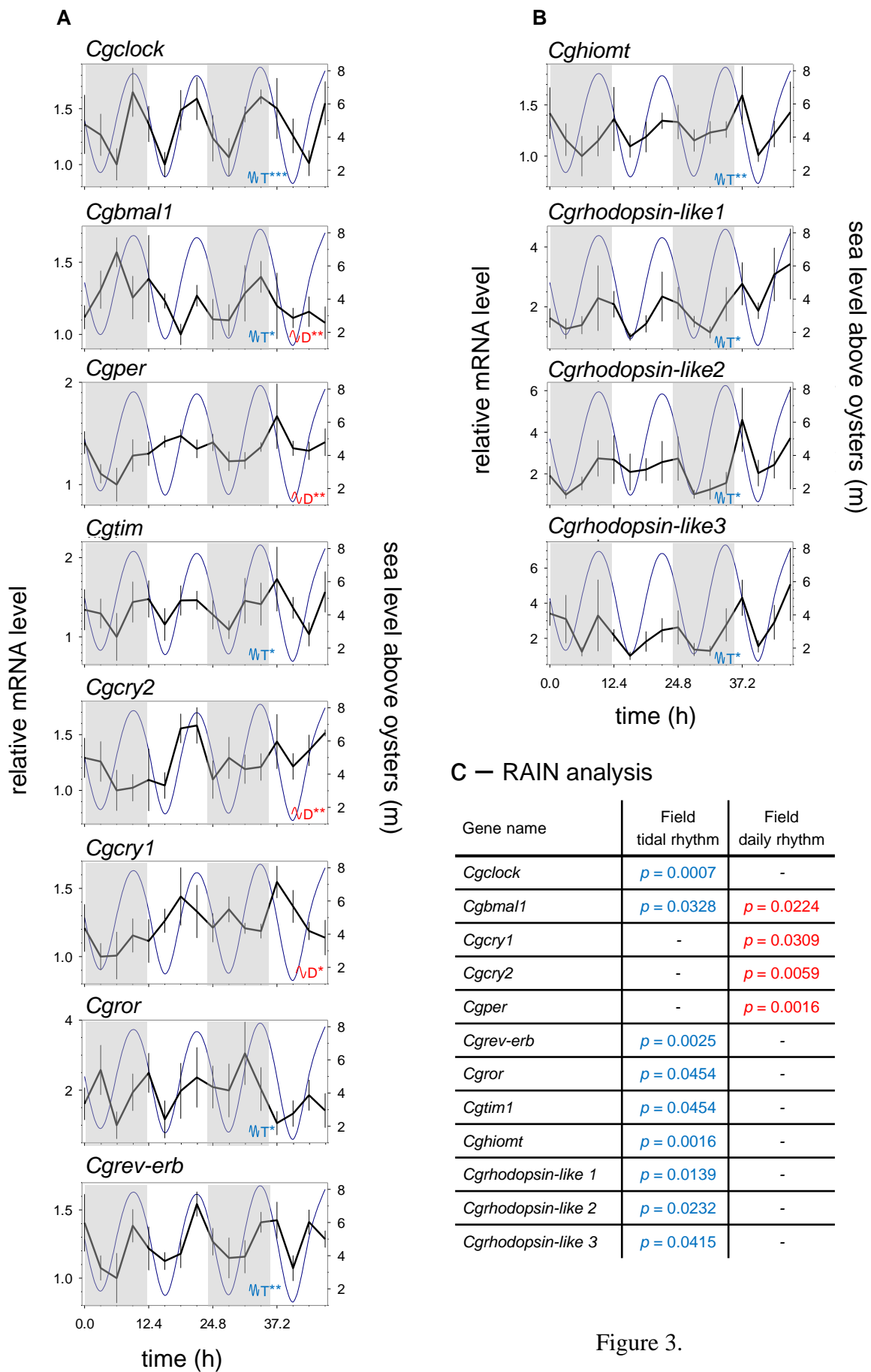
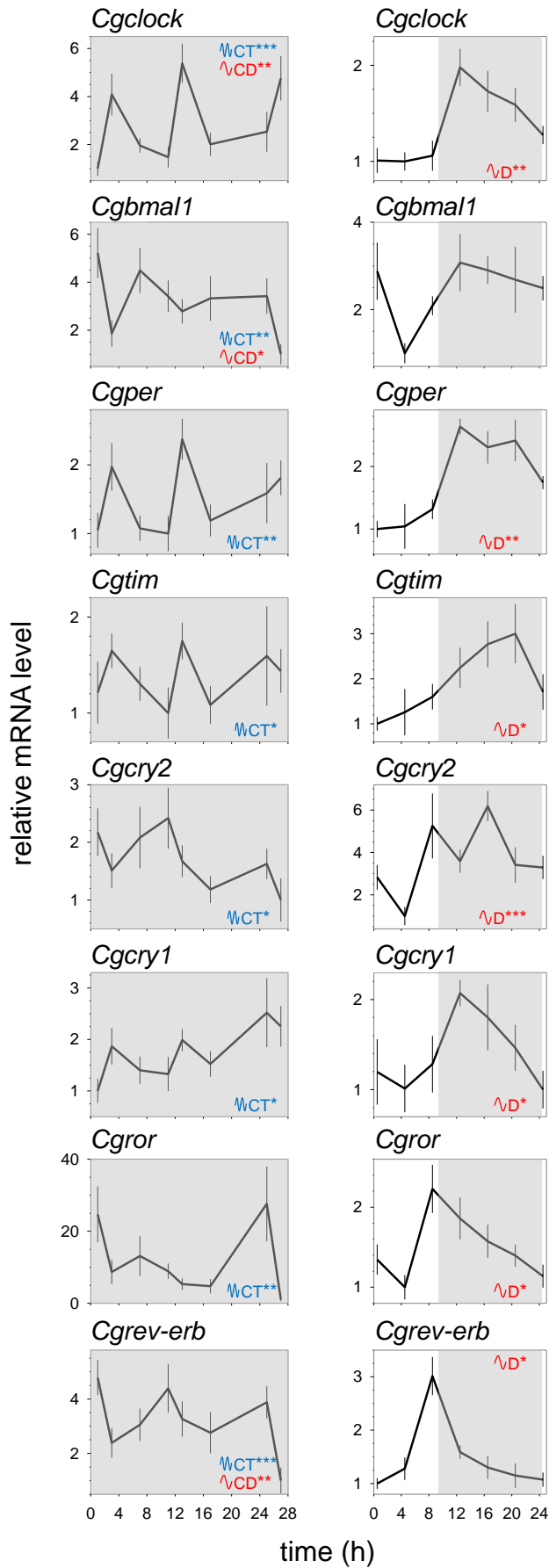


Figure 3.

A – DD expression

B – LD expression

C – RAIN analysis



Gene name	Circatidal rhythm - DD	Circadian rhythm - DD	Circatidal rhythm - LD	Daily rhythm - LD
<i>Cgclock</i>	$p = 0.0007$	$p = 0.0021$	-	$p = 0.0039$
<i>Cgbmal1</i>	$p = 0.0016$	$p = 0.0229$	-	-
<i>Cgcry1</i>	$p = 0.0309$	-	-	$p = 0.0232$
<i>Cgcry2</i>	$p = 0.0102$	-	-	$p = 0.0007$
<i>Cgper</i>	$p = 0.0038$	-	-	$p = 0.0011$
<i>Cgrev-erb</i>	$p = 0.0007$	$p = 0.0052$	-	$p = 0.0292$
<i>Cgror</i>	$p = 0.0088$	-	-	$p = 0.0350$
<i>Cgtim1</i>	$p = 0.0442$	-	-	$p = 0.0165$

Figure 4.